invention. Such a vector would contain a sequence encoding the VRPI antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the VRPI antisense RNA can be by any promoter known in the art to act in mammalian, preferably human, cells. Such promoters can be inducible or constitutive. Examples of such promoters are outlined above.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a gene encoding a VRPI, preferably a human gene encoding a VRPI. However, absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," as referred to herein, means a sequence having sufficient complementarity to be able to hybridize under stringent conditions (*e.g.*, highly stringent conditions comprising hybridization in 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C and washing in 0.1xSSC/0.1% SDS at 68°C, or moderately stringent conditions comprising washing in 0.2xSSC/0.1% SDS at 42°C) with the RNA, forming a stable duplex; in the case of double-stranded VRPI antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA encoding a VRPI it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

5.15.6 Therapeutic Use of VRPI Antisense Nucleic Acids

The VRPI antisense nucleic acids can be used to treat or prevent vascular response when the target VRPI is overexpressed in the blood of subjects suspected of having or suffering from vascular response. In a preferred embodiment, a single-stranded DNA antisense VRPI oligonucleotide is used.

Cell types which express or overexpress RNA encoding a VRPI can be identified by various methods known in the art. Such cell types include but are not limited to leukocytes (*e.g.*, neutrophils, macrophages, monocytes) and resident cells (*e.g.*, astrocytes, glial cells, neuronal cells, and ependymal cells). Such methods include, but are not limited to, hybridization with a VRPI-specific nucleic acid (*e.g.*, by Northern hybridization, dot blot hybridization, *in situ* hybridization), observing the ability of RNA from the cell type to be translated *in vitro* into a VRPI, immunoassay, etc. In a preferred aspect, primary tissue from a subject can be assayed for VRPI expression prior to treatment, *e.g.*, by immunocytochemistry or *in situ* hybridization.

Pharmaceutical compositions of the invention, comprising an effective amount of a VRPI antisense nucleic acid in a pharmaceutically acceptable carrier, can be administered to a subject having vascular response.

The amount of VRPI antisense nucleic acid which will be effective in the treatment of vascular response can be determined by standard clinical techniques.

In a specific embodiment, pharmaceutical compositions comprising one or more VRPI antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, such compositions may be used to achieve sustained release of the VRPI antisense nucleic acids.

5.15.7 Inhibitory Ribozyme andTriple Helix Approaches

In another embodiment, symptoms of vascular response may be ameliorated by decreasing the level of a VRPI or VRPI activity by using gene sequences encoding the VRPI in conjunction with well-known gene "knock-out," ribozyme or triple helix methods to decrease gene expression of a VRPI. In this approach ribozyme or triple helix molecules are used to modulate the activity, expression or synthesis of the gene encoding the VRPI, and thus to ameliorate the symptoms of vascular response. Such molecules may be designed to reduce or inhibit expression of a mutant or non-mutant target gene. Techniques for the production and use of such molecules are well known to those of skill in the art.

Ribozyme molecules designed to catalytically cleave gene mRNA transcripts encoding a VRPI can be used to prevent translation of target gene mRNA and, therefore, expression of the gene product. (See, *e.g.*, PCT International Publication WO90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225).

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. (For a review, see Rossi, 1994, Current Biology 4, 469-471). The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage event. The composition of ribozyme molecules must include one or more sequences complementary to the target gene mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see, *e.g.*, U.S. Patent No. 5,093,246, which is incorporated herein by reference in its entirety.

While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy mRNAs encoding a VRPI, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that

the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Myers, 1995, Molecular Biology and Biotechnology: A Comprehensive Desk Reference, VCH Publishers, New York, (see especially Figure 4, page 833) and in Haseloff and Gerlach, 1988, Nature, 334, 585-591, each of which is incorporated herein by reference in its entirety.

Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the mRNA encoding the VRPI, *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one that occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and that has been extensively described by Thomas Cech and collaborators (Zaug, et al., 1984, Science, 224, 574-578; Zaug and Cech, 1986, Science, 231, 470-475; Zaug, et al., 1986, Nature, 324, 429-433; published International patent application No. WO 88/04300 by University Patents Inc.; Been and Cech, 1986, Cell, 47, 207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in the gene encoding the VRPI.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (*e.g.*, for improved stability, targeting, etc.) and should be delivered to cells that express the VRPI *in vivo*. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous mRNA encoding the VRPI and inhibit translation.

Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficacy.

Endogenous VRPI expression can also be reduced by inactivating or "knocking out" the gene encoding the VRPI, or the promoter of such a gene, using targeted homologous recombination (*e.g.*, see Smithies, et al., 1985, Nature 317:230-234; Thomas and Capecchi, 1987, Cell 51:503-512; Thompson et al., 1989, Cell 5:313-321; and Zijlstra et al., 1989, Nature 342:435-438, each of which is incorporated by reference herein in its entirety). For example, a mutant gene encoding a non-functional VRPI (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous gene (either the coding regions or regulatory regions of the gene encoding the VRPI) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the target gene *in vivo*. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the target gene. Such approaches are particularly suited in the agricultural field where modifications to ES (embryonic stem) cells can be used to generate animal offspring with an inactive target gene (*e.g.*, see Thomas and Capecchi, 1987 and Thompson, 1989, *supra*). However this approach can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors.

Alternatively, the endogenous expression of a gene encoding a VRPI can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the gene (*i.e.*, the gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the gene encoding the VRPI in target cells in the body. (See generally, Helene, 1991, Anticancer Drug Des., 6(6), 569-584; Helene, et al., 1992, Ann. N.Y. Acad. Sci., 660, 27-36; and Maher, 1992, Bioassays 14(12), 807-815).

Nucleic acid molecules to be used in triplex helix formation for the inhibition of transcription should be single stranded and composed of deoxynucleotides. The base

composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC^+ triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, contain a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

In instances wherein the antisense, ribozyme, or triple helix molecules described herein are utilized to inhibit mutant gene expression, it is possible that the technique may so efficiently reduce or inhibit the transcription (triple helix) or translation (antisense, ribozyme) of mRNA produced by normal gene alleles of a VRPI that the situation may arise wherein the concentration of VRPI present may be lower than is necessary for a normal phenotype. In such cases, to ensure that substantially normal levels of activity of a gene encoding a VRPI are maintained, gene therapy may be used to introduce into cells nucleic acid molecules that encode and express the VRPI that exhibit normal gene activity and that do not contain sequences susceptible to whatever antisense, ribozyme, or triple helix treatments are being utilized. Alternatively, in

instances whereby the gene encodes an extracellular protein, normal VRPI can be co-administered in order to maintain the requisite level of VRPI activity.

Antisense RNA and DNA, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules, as discussed above. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

5.16 Assays For Therapeutic Or Prophylactic Compounds

The present invention also provides assays for use in drug discovery in order to identify or verify the efficacy of compounds for treatment or prevention of vascular response. Test compounds can be assayed for their ability to restore VRF or VRPI levels in a subject having vascular response towards levels found in subjects free from vascular response or to produce similar changes in experimental animal models of vascular response. Compounds able to restore VRF or VRPI levels in a subject having vascular response towards levels found in subjects free from vascular response or to produce similar changes in experimental animal models of vascular response can be used as lead compounds for further drug discovery, or used therapeutically. VRF and VRPI expression can be assayed by the Preferred Technology, immunoassays, gel electrophoresis followed by visualization, detection of VRPI activity, or any other method taught herein or known to those skilled in the art. Such assays can be used to screen candidate drugs, in clinical monitoring or in drug development, where abundance of a VRF or VRPI can serve as a surrogate marker for clinical disease.

In various specific embodiments, *in vitro* assays can be carried out with cells representative of cell types involved in a subject's disorder, to determine if a compound has a desired effect upon such cell types.

Compounds for use in therapy can be tested in suitable animal model systems prior to testing in humans, including but not limited to rats, mice, chicken, cows, monkeys, rabbits, etc. For *in vivo* testing, prior to administration to humans, any animal model system known in the art may be used It is also apparent to the skilled artisan that, based upon the present disclosure, transgenic animals can be produced with "knock-out" mutations of the gene or genes encoding one or more VRPIs. A "knock-out" mutation of a gene is a mutation that causes the mutated gene to not be expressed, or expressed in an aberrant form or at a low level, such that the activity associated with the gene product is nearly or entirely absent. Preferably, the transgenic animal is a mammal, more preferably, the transgenic animal is a mouse.

In one embodiment, test compounds that modulate the expression of a VRPI are identified in non-human animals (*e.g.*, mice, rats, monkeys, rabbits, and guinea pigs), preferably non-human animal models for vascular response, expressing the VRPI. In accordance with this embodiment, a test compound or a control compound is administered to the animals, and the effect of the test compound on expression of one or more VRPIs is determined. A test compound that alters the expression of a VRPI (or a plurality of VRPIs) can be identified by comparing the level of the selected VRPI or VRPIs (or mRNA(s) encoding the same) in an animal or group of animals treated with a test compound with the level of the VRPI(s) or mRNA(s) in an untreated animal or group of animals or an animal or group of animals treated with a control compound. Techniques known to those of skill in the art can be used to determine the mRNA and protein levels, for example, *in situ* hybridization. The animals may or may not be sacrificed to assay the effects of a test compound.

In another embodiment, test compounds that modulate the activity of a VRPI or a biologically active portion thereof are identified in non-human animals (*e.g.*, mice, rats, monkeys, rabbits, and guinea pigs), preferably non-human animal models for vascular response, expressing the VRPI. In accordance with this embodiment, a test compound or a control compound is administered to the animals, and the effect of a test compound on the activity of a VRPI is determined. A test compound that alters the activity of a VRPI (or a plurality of VRPIs) can be identified by assaying animals treated with a control compound and animals treated with the test compound. The activity of the VRPI can be assessed by detecting induction of a cellular second messenger of the VRPI (*e.g.*, intracellular Ca2+, diacylglycerol, IP3, etc.), detecting catalytic or enzymatic activity of the VRPI or binding partner thereof, detecting the induction of a reporter gene (*e.g.*, a regulatory element that is responsive to a VRPI of the invention operably linked to a nucleic acid encoding a detectable marker, such as luciferase or green fluorescent protein), or detecting a cellular response (*e.g.*, cellular differentiation or cell proliferation). Techniques known to those of skill in the art can be utilized to detect changes in the activity of a VRPI (see, *e.g.*, U.S. Patent No. 5,401,639, which is incorporated herein by reference).

In yet another embodiment, test compounds that modulate the level or expression of a VRPI (or plurality of VRPIs) are identified in human subjects having vascular response, preferably those having vascular response and most preferably those having severe vascular response. In accordance with this embodiment, a test compound or a control compound is administered to the human subject, and the effect of a test compound on VRPI expression is determined by analyzing the expression of the VRPI or the mRNA encoding the same in a biological sample (*e.g.*, blood, serum, plasma, or urine). A test compound that alters the expression of a VRPI can be identified by comparing the level of the VRPI or mRNA encoding the same in a subject or group of subjects treated with a control compound to that in a subject or group of subjects treated with a test compound. Alternatively, alterations in the expression of a VRPI can be identified by comparing the level of the VRPI or mRNA encoding the same in a

subject or group of subjects before and after the administration of a test compound. Techniques known to those of skill in the art can be used to obtain the biological sample and analyze the mRNA or protein expression. For example, the Preferred Technology described herein can be used to assess changes in the level of a VRPI.

In another embodiment, test compounds that modulate the activity of a VRPI (or plurality of VRPIs) are identified in human subjects having vascular response, preferably those having vascular response and most preferably those with severe vascular response. In this embodiment, a test compound or a control compound is administered to the human subject, and the effect of a test compound on the activity of a VRPI is determined. A test compound that alters the activity of a VRPI can be identified by comparing biological samples from subjects treated with a control compound to samples from subjects treated with the test compound. Alternatively, alterations in the activity of a VRPI can be identified by comparing the activity of a VRPI in a subject or group of subjects before and after the administration of a test compound. The activity of the VRPI can be assessed by detecting in a biological sample (*e.g.*, blood, serum, plasma, or urine) induction of a cellular signal transduction pathway of the VRPI (*e.g.*, intracellular Ca2+, diacylglycerol, IP3, etc.), catalytic or enzymatic activity of the VRPI or a binding partner thereof, or a cellular response, for example, cellular differentiation, or cell proliferation. Techniques known to those of skill in the art can be used to detect changes in the induction of a second messenger of a VRPI or changes in a cellular response. For example, RT-PCR can be used to detect changes in the induction of a cellular second messenger.

In a preferred embodiment, a test compound that changes the level or expression of a VRPI towards levels detected in control subjects (*e.g.*, humans free from vascular response) is selected for further testing or therapeutic use. In another preferred embodiment, a test compound that changes the activity of a VRPI towards the activity found in control subjects (*e.g.*, humans free from vascular response) is selected for further testing or therapeutic use.

In another embodiment, test compounds that reduce the severity of one or more symptoms associated with vascular response are identified in human subjects having vascular response, preferably subjects having vascular response and most preferably subjects with severe vascular response. In accordance with this embodiment, a test compound or a control compound is administered to the subjects, and the effect of a test compound on one or more symptoms of vascular response is determined. A test compound that reduces one or more symptoms can be identified by comparing the subjects treated with a control compound to the subjects treated with the test compound. Techniques known to physicians familiar with vascular response can be used to determine whether a test compound reduces one or more symptoms associated with vascular response. For example, a test compound that reduces aetherosclerosisor vasculitis in a subject having vascular response will be beneficial for treating subjects having vascular response.

In a preferred embodiment, a test compound that reduces the severity of one or more symptoms associated with vascular response in a human having vascular response is selected for further testing or therapeutic use.

5.17 Therapeutic and Prophylactic Compositions and Their Use

The invention provides methods of treatment (and prophylaxis) comprising administering to a subject an effective amount of a compound of the invention. In a preferred aspect, the compound is substantially purified (*e.g.*, substantially free from substances that limit its effect or produce undesired side-effects). The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human. In a specific embodiment, a non-human mammal is the subject.

Formulations and methods of administration that can be employed when the compound comprises a nucleic acid are described above; additional appropriate formulations and routes of administration are described below.

Various delivery systems are known and can be used to administer a compound of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction can be enteral or parenteral and include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved, for example, and not by way of limitation, by local infusion during surgery, topical application, *e.g.*, by injection, by means of a catheter, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection into blood or at the site (or former site) of vascular response.

In another embodiment, the compound can be delivered in a vesicle, in particular a liposome (*see* Langer, 1990, Science 249:1527-1533; Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; *see* generally *ibid.*)

In yet another embodiment, the compound can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald et al., 1980, Surgery 88:507; Saudek et al., 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J., 1983, Macromol. Sci. Rev. Macromol. Chem. 23:61; see also Levy et al., 1985, Science 228:190; During et al., 1989, Ann. Neurol. 25:351; Howard et al., 1989, J. Neurosurg. 71:105). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *e.g.*, the endothelial cells lining the blood vessels, thus requiring only a fraction of the systemic dose (see, *e.g.*, Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (1990, Science 249:1527-1533).

In a specific embodiment where the compound of the invention is a nucleic acid encoding a protein, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of

microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see *e.g.*, Joliot et al., 1991, Proc. Natl. Acad. Sci. USA 88:1864-1868), etc. Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in

"Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the subject. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the compound of the invention which will be effective in the treatment of vascular response can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of

administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each subject's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects (a) approval by the agency of manufacture, use or sale for human administration, (b) directions for use, or both.

6. EXAMPLE: IDENTIFICATION OF PROTEINS DIFFERENTIALLY EXPRESSED IN THE BLOOD IN VASCULAR RESPONSE

Vascular injury, in general, and vasculitis (inflammation and necrosis of the blood vessels) in particular can result when certain drugs are administered. However, there are few reliable diagnostic tests for vasculitis, particularly ones that reliably diagnose such vascular damage at its earliest onset while it is still easily reversible. By treating animals with damage-inducing drugs and then examining their blood plasma or serum for protein concentration changes, we have identified protein markers of vasculitis.

Using the following procedure, proteins in blood samples from control animals and animals having vascular response were separated by isoelectric focusing followed by SDS-PAGE and analyzed. Serial samples were taken over time. Parts 6.1.2 to 6.1.15 (inclusive) of the procedure set forth are hereby designated as the 'Reference Protocol'

6.1 MATERIALS AND METHODS

6.1.1 Vasculitis induced by SKF-95654 treatment.

Groups of adult male Sprague-Dawley rats received a single subcutaneous injection of either 100 mg/kg of SKF 95654 dissolved in DMSO, or DMSO vehicle alone. Groups of 3 animals were sacrificed at 1h, 2h, 4h, or 24h after injection. Tissues were obtained for histopathologic analysis by light microscopy. Plasma was obtained for 2-D gel electrophoretic analysis of proteins.

6.1.2 Sample Preparation

A protein assay (Pierce BCA Cat # 23225) was performed on each serum sample as received. Prior to protein separation, each sample was processed for selective depletion of certain proteins, in order to enhance and simplify protein separation and facilitate analysis by removing proteins that may interfere with or limit analysis of proteins of interest. *See* International Patent Application No. PCT/GB99/01742, filed June 1, 1999, which is incorporated by reference in its entirety, with particular reference to the experimental protocol.

Removal of albumin, haptoglobin, transferrin and immunoglobin G (IgG) from blood ("blood depletion") was achieved by an affinity chromatography purification step in which the sample was passed through a series of Hi-Trap columns containing immobilized antibodies for selective removal of albumin, haptoglobin and transferrin, and protein G for selective removal of immunoglobin G. Two affinity columns in a tandem assembly were prepared by coupling antibodies to protein G-sepharose

contained in Hi-Trap columns (Protein G-Sepharose Hi-Trap columns (1 ml) Pharmacia Cat. No. 17-0404-01). This was done by circulating the following solutions sequentially through the columns: (1) Dulbecco's Phosphate Buffered Saline (Gibco BRL Cat. No. 14190-094); (2) concentrated antibody solution; (3) 200 mM sodium carbonate buffer, pH 8.35; (4) cross-linking solution (200 mM sodium carbonate buffer, pH 8.35, 20 mM dimethylpimelimidate); and (5) 500 mM ethanolamine, 500 mM NaCl. A third (un-derivatised) protein G Hi-Trap column was then attached to the lower end of the tandem column assembly.

The chromatographic procedure was automated using an Akta Fast Protein Liquid Chromatography (FPLC) System such that a series of up to seven runs could be performed sequentially. The samples were passed through the series of 3 Hi-Trap columns in which the affinity chromatography media selectively bind the above proteins thereby removing them from the sample. Fractions (typically 3 ml per tube) were collected of unbound material ("Flowthrough fractions") that eluted through the column during column loading and washing stages and of bound proteins ("Bound/Eluted fractions") that were eluted by step elution with Immunopure Gentle Ag/Ab Elution Buffer (Pierce Cat. No. 21013). The eluate containing unbound material was collected in fractions which were pooled, desalted/concentrated by centrifugal ultrafiltration and stored to await further analysis by 2D PAGE.

A volume of depleted blood containing approximately 300 g of total protein was aliquoted and an equal volume of 10% (w/v) SDS (Fluka 71729), 2.3% (w/v) dithiothreitol (BDH 443852A) was added. The sample was heated at 95°C for 5 mins, and then allowed to cool to 20°C. 125 l of the following buffer was then added to the sample:

8M urea (BDH 452043w)
4% CHAPS (Sigma C3023)
65mM dithiotheitol (DTT)

2% (v/v) Resolytes 3.5-10 (BDH 44338 2x)

This mixture was vortexed, and centrifuged at 13000 rpm for 5 mins at 15°C, and the supernatant was analyzed by isoelectric focusing.

6.1.3 Isoelectric Focusing

Isoelectric focusing (IEF), was performed using the Immobiline7 DryStrip Kit (Pharmacia BioTech), following the procedure described in the manufacturer's instructions, *see* Instructions for Immobiline7 DryStrip Kit, Pharmacia, # 18-1038-63, Edition AB (incorporated herein by reference in its entirety). Immobilized pH Gradient (IPG) strips (18cm, pH 3-10 non-linear strips; Pharmacia Cat. # 17-1235-01) were rehydrated overnight at 20°C in a solution of 8M urea, 2% (w/v) CHAPS, 10mM DTT, 2% (v/v) Resolytes 3.5-10, as described in the Immobiline DryStrip Users Manual. For IEF, 50ml of supernatant (prepared as above) was loaded onto a strip, with the cup-loading units being placed at the basic end of the strip. The loaded gels were then covered with mineral oil (Pharmacia 17-3335-01) and a voltage was immediately applied to the strips according to the following profile, using a Pharmacia EPS3500XL power supply (Cat 19-3500-01):

Initial voltage = 300V for 2 hrs

Linear Ramp from 300V to 3500V over 3hrs

Hold at 3500V for 19hrs

For all stages of the process, the current limit was set to 10mA for 12 gels, and the wattage limit to 5W. The temperature was held at 20°C throughout the run.

6.1.4 Gel Equilibration and SDS-PAGE

After the final 19hr step, the strips were immediately removed and immersed for 10 mins at 20°C in a first solution of the following composition: 6M urea; 2% (w/v) DTT; 2% (w/v) SDS; 30% (v/v) glycerol (Fluka 49767); 0.05M Tris/HCl, pH 6.8 (Sigma Cat T-1503). The strips were removed from the first solution and immersed for 10 mins at

20°C in a second solution of the following composition: 6M urea; 2% (w/v) iodoacetamide (Sigma I-6125); 2% (w/v) SDS; 30% (v/v) glycerol; 0.05M Tris/HCl, pH 6.8. After removal from the second solution, the strips were loaded onto supported gels for SDS-PAGE according to Hochstrasser et al., 1988, Analytical Biochemistry 173: 412-423 (incorporated herein by reference in its entirety), with modifications as specified below.

6.1.5 Preparation of supported gels

The gels were cast between two glass plates of the following dimensions: 23cm wide x 24cm long (back plate); 23cm wide x 24cm long with a 2cm deep notch in the central 19cm (front plate). To promote covalent attachment of SDS-PAGE gels, the back plate was treated with a 0.4% solution of gamma-methacryl-oxypropyltrimethoxysilane in ethanol (BindSilaneJ; Pharmacia Cat. # 17-1330-01). The front plate was treated with (RepelSilaneJ Pharmacia Cat. # 17-1332-01) to reduce adhesion of the gel. Excess reagent was removed by washing with water, and the plates were allowed to dry. At this stage, both as identification for the gel, and as a marker to identify the coated face of the plate, an adhesive bar-code was attached to the back plate in a position such that it would not come into contact with the gel matrix.

The dried plates were assembled into a casting box with a capacity of 13 gel sandwiches. The top and bottom plates of each sandwich were spaced by means of 1mm thick spacers, 2.5 cm wide. The sandwiches were interleaved with acetate sheets to facilitate separation of the sandwiches after gel polymerization. Casting was then carried out according to Hochstrasser et al., *op. cit.*

A 9-16% linear polyacrylamide gradient was cast, extending up to a point 2cm below the level of the notch in the front plate, using the Angelique gradient casting system (Large Scale Biology). Stock solutions were as follows. Acrylamide (40% in water) was from Serva (Cat. # 10677). The cross-linking agent was PDA (BioRad 161-0202),

at a concentration of 2.6% (w/w) of the total starting monomer content. The gel buffer was 0.375M Tris/HCl, pH 8.8. The polymerization catalyst was 0.05% (v/v) TEMED (BioRad 161-0801), and the initiator was 0.1% (w/v) APS (BioRad 161-0700). No SDS was included in the gel and no stacking gel was used. The cast gels were allowed to polymerize at 20°C overnight, and then stored at 4°C in sealed polyethylene bags with 6ml of gel buffer, and were used within 4 weeks.

6.1.6 SDS-PAGE

A solution of 0.5% (w/v) agarose (Fluka Cat 05075) was prepared in running buffer (0.025M Tris, 0.198M glycine (Fluka 50050), 1% (w/v) SDS, supplemented by a trace of bromophenol blue). The agarose suspension was heated to 70°C with stirring, until the agarose had dissolved. The top of the supported 2nd D gel was filled with the agarose solution, and the equilibrated strip was placed into the agarose, and tapped gently with a palette knife until the gel was intimately in contact with the 2nd D gel. The gels were placed in the 2nd D running tank, as described by Amess et al., 1995, Electrophoresis 16: 1255-1267 (incorporated herein by reference in its entirety). The tank was filled with running buffer (as above) until the level of the buffer was just higher than the top of the region of the 2nd D gels which contained polyacrylamide, so as to achieve efficient cooling of the active gel area. Running buffer was added to the top buffer compartments formed by the gels, and then voltage was applied immediately to the gels using a Consort E-833 power supply. For 1 hour, the gels were run at 20mA/gel. The wattage limit was set to 150W for a tank containing 6 gels, and the voltage limit was set to 600V. After 1 hour, the gels were then run at 40mA/gel, with the same voltage and wattage limits as before, until the bromophenol blue line was 0.5cm from the bottom of the gel. The temperature of the buffer was held at 16°C throughout the run. Gels were not run in duplicate.

6.1.7 Staining

Upon completion of the electrophoresis run, the gels were immediately removed from the tank for fixation. The top plate of the gel cassette was carefully removed, leaving

the gel bonded to the bottom plate. The bottom plate with its attached gel was then placed into a staining apparatus, which can accommodate 12 gels. The gels were completely immersed in fixative solution of 40% (v/v) ethanol (BDH 28719), 10% (v/v) acetic acid (BDH 100016X), 50% (v/v) water (MilliQ-Millipore), which was continuously circulated over the gels. After an overnight incubation, the fixative was drained from the tank, and the gels were primed by immersion in 7.5% (v/v) acetic acid, 0.05% (w/v) SDS, 92.5% (v/v) water for 30 mins. The priming solution was then drained, and the gels were stained by complete immersion for 4 hours in a staining solution of Pyridinium, 4-[2-[4-(dipentylamino)-2-trifluoromethylphenyl] ethenyl]-1-(sulfobutyl)-, inner salt, prepared by diluting a stock solution of this dye (2mg/ml in DMSO) in 7.5% (v/v) aqueous acetic acid to give a final concentration of 1.2 mg/l; the staining solution was vacuum filtered through a 0.4 m filter (Duropore) before use.

6.1.8 Imaging of the gel

A computer-readable output was produced by imaging the fluorescently stained gels with the Apollo 2 scanner (Oxford Glycosciences, Oxford, UK) described in section 5.1, *supra*. This scanner has a gel carrier with four integral fluorescent markers (Designated M1, M2, M3, M4) that are used to correct the image geometry and are a quality control feature to confirm that the scanning has been performed correctly.

For scanning, the gels were removed from the stain, rinsed with water and allowed to air dry briefly, and imaged on the Apollo 2. After imaging, the gels were sealed in polyethylene bags containing a small volume of staining solution, and then stored at 4°C.

6.1.9 Digital Analysis of the Data

The data were processed as described in U.S. Patent No. 6,064,754 at Sections 5.4 and 5.5 (incorporated herein by reference), as set forth more particularly below.

The output from the scanner was first processed using the MELANIE7 II 2D PAGE analysis program (Release 2.2, 1997, BioRad Laboratories, Hercules, California, Cat. # 170-7566) to autodetect the registration points, M1, M2, M3 and M4; to autocrop the images (*i.e.*, to eliminate signals originating from areas of the scanned image lying outside the boundaries of the gel, *e.g.* the reference frame); to filter out artifacts due to dust; to detect and quantify features; and to create image files in GIF format. Features were detected using the following parameters:

Smooths =2

Laplacian threshold 50

Partials threshold 1

Saturation = 100

Peakedness = 0

Minimum Perimeter = 10

6.1.10 Assignment of pI and MW Values

Landmark identification was used to determine the pI and MW of features detected in the images. Twelve landmark features, designated F1 to F11 and F13, were identified in a standard blood image obtained from a pooled sample. These landmark features are identified in Figure 2 and were assigned the pI and/or MW values identified in Table IX.

Table IX. Landmark Features Used In This Study

Name	pI	MW (Da)	Name	PI	MW (Da)
F1	-1	32208	F7	5.31	133062
F2	4.98	57602	F8	-1	10505

F3	5.64	49312	F9	-1	15241
F4	6.35	18635	F10	9.25	25534
F5	5.92	36216	F11	6.06	66164
F6	7.16	74162	F13	4.22	12236

As many of these landmarks as possible were identified in each gel image of the dataset. Each feature in the study gels was then assigned a pI value by linear interpolation or extrapolation (using the MELANIE7-II software) to the two nearest landmarks, and was assigned a MW value by linear interpolation or extrapolation (using the MELANIE7-II software) to the two nearest landmarks.

6.1.11 Matching With Primary Master Image

Images were edited to remove gross artifacts such as dust, to reject images which had gross abnormalities such as smearing of protein features, or were of too low a loading or overall image intensity to allow identification of more than the most intense features, or were of too poor a resolution to allow accurate detection of features. Images were then compared by pairing with one common image from the whole sample set. This common image, the "primary master image", was selected on the basis of protein load (maximum load consistent with maximum feature detection), and general image quality. Additionally, the primary master image was chosen to be an image which appeared to be generally representative of all those to be included in the analysis. (This process by which a primary master gel was judged to be representative of the study gels was rechecked by the method described below and in the event that the primary master gel was seen to be unrepresentative, it was rejected and the process repeated until a representative primary master gel was found).

Each of the remaining study gel images was individually matched to the primary master image such that common protein features were paired between the primary master image and each individual study gel image as described below.

6.1.12 Cross-matching Between Samples

To facilitate statistical analysis of large numbers of samples for purposes of identifying features that are differentially expressed, the geometry of each study gel was adjusted for maximum alignment between its pattern of protein features, and that of the primary master, as follows. Each of the study gel images was individually transformed into the geometry of the primary master image using a multi-resolution warping procedure. This procedure corrects the image geometry for the distortions brought about by small changes in the physical parameters of the electrophoresis separation process from one sample to another. The observed changes are such that the distortions found are not simple geometric distortions, but rather a smooth flow, with variations at both local and global scale.

The fundamental principle in multi-resolution modeling is that smooth signals may be modeled as an evolution through 'scale space', in which details at successively finer scales are added to a low resolution approximation to obtain the high resolution signal. This type of model is applied to the flow field of vectors (defined at each pixel position on the reference image) and allows flows of arbitrary smoothness to be modeled with relatively few degrees of freedom. Each image is first reduced to a stack, or pyramid, of images derived from the initial image, but smoothed and reduced in resolution by a factor of 2 in each direction at every level (Gaussian pyramid) and a corresponding difference image is also computed at each level, representing the difference between the smoothed image and its progenitor (Laplacian pyramid). Thus the Laplacian images represent the details in the image at different scales.

To estimate the distortion between any 2 given images, a calculation was performed at level 7 in the pyramid (i.e. after 7 successive reductions in resolution). The Laplacian images were segmented into a grid of 16x16 pixels, with 50% overlap between adjacent grid positions in both directions, and the cross correlation between corresponding grid squares on the reference and the test images was computed. The distortion displacement was then given by the location of the maximum in the

correlation matrix. After all displacements had been calculated at a particular level, they were interpolated to the next level in the pyramid, applied to the test image, and then further corrections to the displacements were calculated at the next scale.

The warping process brought about good alignment between the common features in the primary master image, and the images for the other samples. The MELANIE7 II 2D PAGE analysis program was used to calculate and record approximately 500-700 matched feature pairs between the primary master and each of the other images. The accuracy of this program was significantly enhanced by the alignment of the images in the manner described above. To improve accuracy still further, all pairings were finally examined by eye in the MelView interactive editing program and residual recognizably incorrect pairings were removed. Where the number of such recognizably incorrect pairings exceeded the overall reproducibility of the Preferred Technology (as measured by repeat analysis of the same biological sample) the gel selected to be the primary master gel was judged to be insufficiently representative of the study gels to serve as a primary master gel. In that case, the gel chosen as the primary master gel was rejected, and different gel was selected as the primary master gel, and the process was repeated.

All the images were then added together to create a composite master image, and the positions and shapes of all the gel features of all the component images were super-imposed onto this composite master as described below.

Once all the initial pairs had been computed, corrected and saved, a second pass was performed whereby the original (unwarped) images were transformed a second time to the geometry of the primary master, this time using a flow field computed by smooth interpolation of the multiple tie-points defined by the centroids of the paired gel features. A composite master image was thus generated by initializing the primary master image with its feature descriptors. As each image was transformed into the primary master geometry, it was digitally summed pixel by pixel into the composite

master image, and the features that had not been paired by the procedure outlined above were likewise added to the composite master image description, with their centroids adjusted to the master geometry using the flow field correction.

The final stage of processing was applied to the composite master image and its feature descriptors, which now represent all the features from all the images in the study transformed to a common geometry. The features were grouped together into linked sets or "clusters", according to the degree of overlap between them. Each cluster was then given a unique identifying index, the molecular cluster index (MCI).

An MCI identifies a set of matched features on different images. Thus an MCI represents a protein or proteins eluting at equivalent positions in the 2D separation in different samples.

6.1.13 Construction of Profiles

After matching all component gels in the study to the final composite master image, the intensity of each feature was measured and stored. The end result of this analysis was the generation of a digital profile which contained, for each identified feature: 1) a unique identification code relative to corresponding feature within the composite master image (MCI), 2) the x, y coordinates of the features within the gel, 3) the isoelectric point (pI) of the VRFs, 4) the apparent molecular weight (MW) of the VRFs, 5) the signal value, 6) the standard deviation for each of the preceding measurements, and 7) a method of linking the MCI of each feature to the master gel to which this feature was matched. By virtue of a Laboratory Information Management System (LIMS), this MCI profile was traceable to the actual stored gel from which it was generated, so that proteins identified by computer analysis of gel profile databases could be retrieved. The LIMS also permitted the profile to be traced back to an original sample or patient.

6.1.14 Statistical Analysis of the Profiles

Comparisons were made between samples taken from control subjects and samples taken from subjects having vascular response induced by SKF-95654 treatment for 1 hour, 2 hours, 4 hours and 24 hours.

The statistical strategies specified used to identify VRFs from the MCIs within the mastergroup is based on qualitative presence or absence alone. Using this procedure, a percentage feature presence was calculated across the control samples and vascular response samples for each MCI which was a potential VRF based on such qualitative criteria alone, i.e. presence or absence. Feature Presence Threshold Percentage values of 66 or 66 (MCI present in either 66 % of foreground or background samples), and 100 or 100 (MCI present in either 100 % of foreground or background samples) were separately applied together with a 98 % confidence limit. The MCIs which recorded a percentage feature presence of 98% or more on vascular response samples and a percentage feature presence of 2% or less on control samples, were selected as the qualitative differential VRFs with 98% selectivity.

MCIs with significant fold changes were selected for each of the MCIs chosen in the first instance by feature presence.

A second selection strategy was based on the fold change. A fold change representing the ratio of the average normalized protein abundances of the CRF /MCI, was calculated for each MCI between each set of controls and treated samples. A 95% confidence limit for the mean of the fold changes was calculated. The MCIs with fold changes which fall outside the confidence limit were selected as CRFs which met the criteria of the significant fold change threshold with 95% selectivity. Because the MCI fold changes are based on a 95% confidence limit, it follows that the significant fold change threshold is itself 95%. A parametric test (T-test) was applied. This test was performed between the control and the treated samples for each MCI. The MCIs which recorded a p-value less than or equal to 0.05 were selected as statistically significant CRFs with 95% selectivity. The MCIs which recorded a p-value less than or equal to

0.01 were selected as statistically significant CRFs with 99% selectivity. The MCIs which recorded a p-value less than or equal to 0.001 were selected as statistically significant CRFs with 99.9% selectivity.

Application of this analysis strategy allowed CRFs to be selected on the basis of: a significant fold change 2.0 with a chosen selectivity (p-value 0.05, or 0.01 or 0.001).

6.1.15 Recovery and analysis of selected proteins

Proteins in VRFs were robotically excised and processed to generate tryptic digest peptides. Tryptic peptides were analyzed by mass spectrometry using a PerSeptive Biosystems Voyager- DE™ STR Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometer, and selected tryptic peptides were analyzed by tandem mass spectrometry (MS/MS) using a Micromass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (Micromass, Altrincham, U.K.) equipped with a nanoflow™ electrospray Z-spray source. For partial amino acid sequencing and identification of VRPIs uninterpreted tandem mass spectra of tryptic peptides were searched using the SEQUEST search program (Eng et al., 1994, J. Am. Soc. Mass Spectrom. 5:976-989), version v.C.1. Criteria for database identification included: the cleavage specificity of trypsin; the detection of a suite of a, b and y ions in peptides returned from the database, and a mass increment for all Cys residues to account for carbamidomethylation. The database searched was database constructed of protein entries in the non-redundant database held by the National Centre for Biotechnology Information (NCBI) which is accessible at http://www.ncbi.nlm.nih.gov/. Following identification of proteins through spectral-spectral correlation using the SEQUEST program, masses detected in MALDI-TOF mass spectra were assigned to tryptic digest peptides within the proteins identified. In cases where no amino acid sequences could be identified through searching with uninterpreted MS/MS spectra of tryptic digest peptides using the SEQUEST program, tandem mass spectra of the peptides were interpreted manually, using methods known in the art. (In the case of interpretation of

low-energy fragmentation mass spectra of peptide ions see Gaskell et al., 1992, Rapid Commun. Mass Spectrom. 6:658-662)

6.2 Results

Lesions were seen in the mesenteric vasculature 24h after dosing with the SKF 95654 but not in DMSO treated animals. The lesions consisted mainly of arterial medial hemorrhage, and venous thrombosis and vasculitis in arteries, in terminal arterioles, in capillaries, in postcapillary venules, and in small veins. Light and electron microscopic evaluation showed the presence of endothelial and smooth muscle necrosis in affected vessels. In addition TUNEL (terminal uridinedeoxynucleotide end labeling) staining for apoptosis, and electron microscopic analysesdetected both vascular endothelial cell and vascular smooth muscle cell apoptosis at 1h, 2h, and 4 h after treatment with SKF 95654. Electron microscopy also detected ultrastructural alterations consistent with vascular endothelial cell activation.

These initial experiments identified 73 features that were decreased and 194 features that were increased in Vascular Response plasma as compared with normal plasma. Details of these VRFs are provided in Tables I and II and X. Each VRF was differentially present in Vascular Response plasma as compared with normal plasma. Partial amino acid sequences were determined for the VRPIs present in these VRFs. Details of these VRPIs are provided in Tables IV and V.

Table X lists the VRFs that were differentially present in SKF-95654 (SKF) treated subjects versus control/non-treated subjects. The fold change observed over 1, 2, 4 and 24 hours is shown. The first half of the table lists VRFs that were decreased, and the second half lists VRFs that were increased, upon SKF treatment.

Table X Differentially expressed VRFs in Vascular Response

Table X			Fold Change (SKF(h) vs DMSO 24h)				P-Value, T-Test, (SKF(h) vs DMSO 24h)			
VRF	PI	MW (Da)	SKF (1h)	SKF (2h)	SKF (4h)	SKF (24h)	SKF (1h)	SKF (2h)	SKF (4h)	SKF (24h)
VRF-1	6.9	55,862	-92.60	-92.60	-95.30	-92.60				
VRF-2	4.5	12,427	-28.20	-34.02	-42.71	-21.27	0.028	0.028	0.027	0.028
VRF-3	5.9	53,798	-28.20			-28.20				
VRF-4	6.6	55,660	-14.22	-17.16	-21.54	-17.72	0.028	0.028	0.027	0.027
VRF-5	5.4	66,982	-24.70							
VRF-6	6.3	59,720	-17.30							
VRF-7	7.5	45,196	-11.50	-11.50	-11.80	-11.50				
VRF-8	5.0	36,628	-7.10	-19.10	-25.20	-3.90				
VRF-9	6.8	38,561	-8.27				0.040			
VRF-10	4.6	53,996	-5.20							
VRF-11	6.4	43,053	-2.42		-3.67		0.010		0.004	
VRF-12	7.0	50,908	-4.37	-9.07	-5.63	-4.70	0.009	0.012	0.020	0.018
VRF-13	5.3	36,415		-56.80	-287.40	-3.80				
VRF-14	5.1	36,546		-51.50	-34.90	-4.00				
VRF-15	7.6	40,071	-4.23	-25.38	-5.64		0.040	0.028	0.021	
VRF-16	5.9	93,545		-37.00	-5.70					
VRF-17	7.2	45,318	-3.35	-12.02	-15.09	-7.33	0.015	0.013	0.012	0.004
VRF-18	5.5	35,251		-15.80	-11.00					
VRF-19	5.4	30,705		-10.30	-10.60					
VRF-20	5.8	88,662		-8.60	-8.90	-8.60				
VRF-21	6.4	30,462		-5.35	-6.72	-4.33		0.025	0.022	0.012
VRF-22	5.6	117,048		-7.80						
VRF-23	5.3	146,763		-6.80	-5.20					
VRF-24	6.9	25,788		-6.60						
VRF-25	7.5	36,779		-9.17				0.045		
VRF-26	6.4	22,672		-5.20						
VRF-27	6.4	57,270		-5.10						
VRF-28	5.0	31,998	-6.70	-11.28	-39.36	-32.38	0.018	0.023	0.029	0.029
VRF-28	5.0	31,998	-6.70	-11.28	-39.36	-32.38	0.018	0.023	0.029	0.029
VRF-29	6.1	103,294		-4.50						
VRF-30	6.3	55,313		-6.72	-46.93			0.008	0.004	
VRF-31	5.6	89,343			-21.40					
VRF-32	7.5	47,734	-5.31		-15.70		0.003		0.007	
VRF-32	7.5	47,734	-5.31		-15.70		0.003		0.007	
VRF-34	6.3	114,395	-3.12	-3.88	-7.09	-6.30	0.015	0.005	0.001	0.001
VRF-35	4.7	15,304			-4.80					
VRF-36	4.9	67,894			-4.70					
VRF-37	6.7	23,985				-14.00				
VRF-38	5.9	51,173				-10.60				
VRF-40	6.6	109,011	-4.05		-6.49	-5.81	0.029		0.019	0.034
VRF-42	4.6	10,631				-4.50				

Table X			Fold Change (SKF(h) vs DMSO 24h)				P-Value, T-Test, (SKF(h) vs DMSO 24h)			
VRF	PI	MW (Da)	SKF (1h)	SKF (2h)	SKF (4h)	SKF (24h)	SKF (1h)	SKF (2h)	SKF (4h)	SKF (24h)
VRF-131	8.6	62,760	-11.71				0.042			
VRF-132	8.6	56,316	-9.70	-3.15	-4.84	-8.50	0.016	0.036	0.022	0.015
VRF-133	6.1	170,804	-5.42	-41.38	-51.95	-42.74	0.019	0.021	0.021	0.021
VRF-134	5.5	106,240	-4.52				0.047			
VRF-135	6.4	76,184	-4.36				0.032			
VRF-136	6.6	56,091	-3.69	-3.55			0.012	0.003		
VRF-137	5.3	108,420	-3.58				0.020			
VRF-138	6.2	161,240	-3.55		-2.10		0.016		0.020	
VRF-139	6.3	56,572	-3.41				0.002			
VRF-140	8.4	47,387	-3.26				0.037			
VRF-141	6.0	159,928	-2.90				0.036			
VRF-142	5.3	40,795	-2.84				0.022			
VRF-143	8.0	46,894	-2.74				0.046			
VRF-144	5.2	104,122	-2.68				0.044			
VRF-145	6.2	47,376	-2.40		-4.50		0.015		0.015	
VRF-146	5.5	48,580	-2.29				0.041			
VRF-147	7.2	57,090	-2.27				0.006			
VRF-148	7.4	51,981	-2.24	-2.95			0.001	0.048		
VRF-149	6.9	57,970	-2.21				0.022			
VRF-150	7.3	54,641	-2.15	-3.28			0.013	0.025		
VRF-151	7.2	74,287	-2.14				0.011			
VRF-152	6.7	108,339	-2.13		-2.09	-2.10	0.023		0.005	0.006
VRF-183	5.2	90,873		-7.31	-76.28	-7.36		0.008	0.004	0.008
VRF-184	6.8	35,881		-2.86				0.046		
VRF-185	6.9	47,790		-2.82				0.043		
VRF-186	6.3	33,687		-2.50				0.050		
VRF-187	6.3	33,460		-2.34	-2.32			0.032	0.019	
VRF-188	6.6	29,779		-2.30				0.035		
VRF-226	5.3	102,172			-5.53				0.034	
VRF-227	5.5	163,669			-4.52				0.005	
VRF-228	6.3	160,221			-2.00	-4.86			0.004	0.001
VRF-229	6.4	66,374			-2.00				0.038	
VRF-246	6.4	34,560				-2.22				0.046
VRF-153	5.9	164,081	-2.10	2.09			0.029	0.003		
VRF-43	5.3	15,671	6.00							
VRF-44	5.7	42,612	6.10							
VRF-45	6.6	65,153	6.20							
VRF-46	4.6	26,025	6.40							
VRF-47	5.8	63,791	7.20							
VRF-47	5.8	63,791	7.20							
VRF-48	7.5	41,646	7.30	5.70	7.70					
VRF-50	6.5	28,210	7.90							
VRF-51	6.9	46,197	8.70							
VRF-52	7.3	14,777	9.50							

Table X			Fold Change (SKF(h) vs DMSO 24h)				P-Value, T-Test, (SKF(h) vs DMSO 24h)			
VRF	PI	MW (Da)	SKF (1h)	SKF (2h)	SKF (4h)	SKF (24h)	SKF (1h)	SKF (2h)	SKF (4h)	SKF (24h)
VRF-53	5.7	24,740	12.00							
VRF-54	6.0	54,784	9.54				0.002			
VRF-55	4.7	15,983	18.30							
VRF-56	5.5	46,824	12.57	9.77	9.63		0.036	2.9E-06	0.002	
VRF-57	5.4	41,990	19.00							
VRF-58	4.8	42,404	19.40							
VRF-59	4.8	49,983	20.50							
VRF-60	6.7	65,458	20.50							
VRF-61	5.6	41,149	20.70							
VRF-62	6.2	80,775		11.07	21.88	15.82		0.030	0.041	0.044
VRF-63	6.0	89,519	22.10							
VRF-64	5.9	55,255			3.90				3.4E-04	
VRF-65	5.6	23,764	24.10		14.40					
VRF-66	6.0	52,425				27.31				0.036
VRF-67	7.4	12,797	30.90							
VRF-68	5.0	32,691	21.54	22.77	33.81		0.014	0.026	0.008	
VRF-68	5.0	32,691	21.54	22.77	33.81		0.014	0.026	0.008	
VRF-69	6.3	52,057			30.25				0.005	
VRF-70	4.4	35,889		50.03	42.93	39.71		0.015	0.033	0.001
VRF-71	7.0	72,009	46.90							
VRF-72	5.3	46,549	94.80	60.50						
VRF-73	5.3	56,876			109.98				0.008	
VRF-74	4.7	31,099	123.30							
VRF-75	5.5	51,088	140.80							
VRF-76	5.3	41,608	119.23		95.01		0.023		0.025	
VRF-77	5.9	106,831		4.20						
VRF-79	4.5	28,816		6.26		4.47		0.004		0.015
VRF-80	5.8	109,095		4.60						
VRF-81	6.8	27,783		3.34				0.018		
VRF-82	5.5	44,702			2.82				0.012	
VRF-83	4.5	35,312		7.81	6.20	6.49		0.015	0.011	0.008
VRF-84	5.7	110,078			3.94	3.43			0.013	0.033
VRF-85	5.2	118,045		8.40						
VRF-86	5.9	65,200			3.26				0.045	
VRF-87	6.3	53,617		10.80	28.10					
VRF-88	7.6	47,020		11.80	35.40					
VRF-89	4.9	39,092		12.90						
VRF-90	7.2	46,463			14.05	14.17			0.037	0.040
VRF-91	4.4	36,411		17.50	15.40	8.70				
VRF-92	5.5	64,501		12.00				0.002		
VRF-93	4.6	71,770		25.30						
VRF-94	5.0	49,783		17.28				0.000		
VRF-95	5.3	23,986		37.70						
VRF-96	7.0	56,480		34.73		34.10		0.006		0.019

Table X			Fold Change (SKF(h) vs DMSO 24h)				P-Value, T-Test, (SKF(h) vs DMSO 24h)			
VRF	PI	MW (Da)	SKF (1h)	SKF (2h)	SKF (4h)	SKF (24h)	SKF (1h)	SKF (2h)	SKF (4h)	SKF (24h)
VRF-97	6.6	51,988			66.76				0.003	
VRF-98	4.6	79,952		38.01				0.039		
VRF-99	4.6	70,686		39.82				0.019		
VRF-100	7.0	53,025		173.59	212.14			0.025	0.011	
VRF-101	5.0	88,760			4.27				0.009	
VRF-102	5.1	75,937			4.40					
VRF-103	7.5	27,997		2.42				0.044		
VRF-104	4.9	45,028			4.90					
VRF-105	4.7	30,156			5.20					
VRF-106	5.2	23,929	2.65	2.77			0.043	0.018		
VRF-107	6.4	103,089		6.04				0.030		
VRF-108	6.1	94,872			5.80	4.10				
VRF-109	6.5	44,363			4.62	6.10			0.017	0.013
VRF-110	6.6	117,961			5.61				0.010	
VRF-111	5.0	12,909			11.10					
VRF-112	6.0	47,782			10.58	15.65			0.034	0.050
VRF-113	6.3	24,290			16.20					
VRF-114	5.5	62,780			20.60					
VRF-115	5.0	35,008			27.40					
VRF-116	7.2	50,820			21.65				0.001	
VRF-117	7.8	55,125			24.45				0.021	
VRF-118	5.0	61,654			57.90					
VRF-118	5.0	61,654			57.90					
VRF-119	5.3	36,415			71.90	49.70				
VRF-120	4.8	67,581				7.79				0.026
VRF-122	5.9	18,986				6.00				
VRF-123	6.3	49,733				5.02				0.038
VRF-124	4.7	68,327				7.80				
VRF-125	5.3	27,768				7.90				
VRF-126	6.1	57,486				7.73				0.022
VRF-127	6.2	54,118				9.39				0.008
VRF-128	7.6	24,430				14.60				
VRF-129	5.0	52,708				59.63				0.032
VRF-130	7.6	52,327				808.10				
VRF-154	5.7	93,937	2.02				0.024			
VRF-155	5.8	98,012	2.12				0.001			
VRF-156	5.9	46,795	2.14	2.52	2.01		0.023	0.010	0.033	
VRF-157	5.0	71,952	2.20	2.57			0.043	0.045		
VRF-158	5.9	38,180	2.36				0.016			
VRF-159	5.0	86,263	2.56		2.11		0.033		0.004	
VRF-160	4.9	57,857	2.56	2.05			0.036	0.030		
VRF-161	5.3	61,350	2.69				0.026			
VRF-162	5.8	92,965	2.70				0.006			
VRF-163	4.9	42,639	2.83	2.60			0.014	0.050		

Table X			Fold Change (SKF(h) vs DMSO 24h)				P-Value, T-Test, (SKF(h) vs DMSO 24h)			
VRF	PI	MW (Da)	SKF (1h)	SKF (2h)	SKF (4h)	SKF (24h)	SKF (1h)	SKF (2h)	SKF (4h)	SKF (24h)
VRF-164	6.9	41,691	3.29				0.035			
VRF-165	4.5	17,273	3.57	3.50			0.020	0.018		
VRF-166	5.2	53,618	3.62				0.035			
VRF-167	5.5	15,280	3.74				0.034			
VRF-168	5.9	22,620	3.78	2.83			0.008	0.026		
VRF-169	5.1	21,015	4.21				0.028			
VRF-170	5.7	25,824	4.74				0.009			
VRF-171	4.9	73,274	4.76	2.95		2.36	0.003	0.022		0.023
VRF-172	7.8	26,158	4.91				0.013			
VRF-173	5.1	64,281	5.28				0.013			
VRF-174	5.6	12,587	5.30				0.027			
VRF-175	5.5	13,378	6.97	5.80	11.15	8.00	0.023	0.045	0.029	0.002
VRF-176	6.3	12,252	8.18		4.94	5.02	0.003		0.031	0.016
VRF-177	4.9	94,792	9.02				0.017			
VRF-178	5.2	63,354	9.46				0.005			
VRF-179	4.8	46,315	9.83	11.59			0.013	0.006		
VRF-180	6.3	80,457	14.14				0.024			
VRF-181	7.6	11,178	14.38				0.008			
VRF-182	8.0	10,939	233.13				0.031			
VRF-189	7.0	26,002		2.17				0.047		
VRF-190	5.5	26,171		2.25				0.025		
VRF-191	4.5	34,811		2.28		2.16		0.001		0.007
VRF-192	7.5	77,234		2.29				0.005		
VRF-193	5.2	44,506		2.31	3.11			0.039	0.011	
VRF-194	7.5	26,138		2.39	2.47			0.008	0.012	
VRF-195	5.0	64,764		2.61				0.046		
VRF-196	4.4	85,200		2.62		3.30		0.037		0.001
VRF-197	5.0	113,373		2.84				0.019		
VRF-198	6.2	102,667		3.05				0.029		
VRF-199	4.5	31,524		3.29		2.73		0.001		0.002
VRF-200	4.7	28,230		3.51				0.040		
VRF-201	4.5	57,154		3.53	2.38	2.11		0.036	0.035	0.033
VRF-202	5.5	56,331		3.58				0.024		
VRF-203	5.1	79,689		3.61	2.53	3.65		0.038	0.028	0.011
VRF-204	4.8	73,028		3.73				0.035		
VRF-205	4.7	76,949		4.30	4.17			0.036	0.046	
VRF-206	7.6	91,970		4.45				0.035		
VRF-207	4.6	21,586		4.55				0.020		
VRF-208	4.7	59,670		4.64				0.050		
VRF-209	7.7	76,246		4.71				0.036		
VRF-210	7.4	70,172		4.92				0.037		
VRF-211	7.0	23,768		5.29				0.008		
VRF-212	5.8	166,718		5.42				0.011		
VRF-213	4.4	86,497		5.63				0.031		

Table X			Fold Change (SKF(h) vs DMSO 24h)				P-Value, T-Test, (SKF(h) vs DMSO 24h)			
VRF	PI	MW (Da)	SKF (1h)	SKF (2h)	SKF (4h)	SKF (24h)	SKF (1h)	SKF (2h)	SKF (4h)	SKF (24h)
VRF-214	9.2	56,906		5.64				0.034		
VRF-215	4.8	23,997		5.81				0.012		
VRF-216	7.7	70,910		6.03				2.7E-05		
VRF-217	4.5	47,550		6.04				0.051		
VRF-218	7.9	90,621		6.07	5.50	5.93		0.011	0.002	0.041
VRF-219	6.1	127,699		6.71				0.001		
VRF-220	5.5	172,394		9.45				0.002		
VRF-221	4.6	50,897		10.54				0.003		
VRF-222	4.2	12,107		15.14				0.048		
VRF-223	8.1	71,265		19.14	27.58	26.52		0.012	0.040	0.027
VRF-224	4.8	41,462		19.80				0.035		
VRF-225	4.3	20,955		21.28	14.74	30.85		0.022	0.045	0.001
VRF-230	5.1	55,780			2.06				0.040	
VRF-231	5.3	30,594			2.08				0.035	
VRF-232	6.8	86,976			2.13				0.005	
VRF-233	5.1	53,782			2.32				0.017	
VRF-234	6.1	12,243			2.33				0.037	
VRF-235	5.0	34,090			3.02				0.041	
VRF-236	5.1	24,090			3.10				0.044	
VRF-237	5.2	41,154			3.25	2.85			0.049	0.047
VRF-238	4.5	60,771			4.11	6.57			0.051	0.009
VRF-239	5.4	89,494			4.56	6.03			0.040	0.045
VRF-240	7.9	34,448			5.51				0.016	
VRF-241	4.2	45,046			5.55	8.76			0.026	0.009
VRF-242	8.3	46,524			9.17				0.005	
VRF-243	4.7	129,281			28.06				0.026	
VRF-244	6.7	84,357			41.83				0.030	
VRF-245	6.9	87,338			47.32				0.020	
VRF-247	6.3	33,925				2.00				0.002
VRF-248	6.1	27,002				2.05				0.051
VRF-249	7.9	77,468				2.21				0.038
VRF-250	4.4	70,668				2.24				0.027
VRF-251	4.3	10,895				2.30				0.002
VRF-252	4.2	41,478				2.36				0.022
VRF-253	6.0	56,223				2.40				0.030
VRF-254	7.5	40,506				2.47				0.017
VRF-255	8.1	52,364				2.48				0.043
VRF-256	5.7	83,735				2.81				0.046
VRF-257	6.0	12,254				2.90				0.049
VRF-258	4.2	12,240				3.62				0.028
VRF-259	6.5	23,684				3.64				0.009
VRF-260	6.2	60,551				3.69				0.044
VRF-261	6.1	23,907				4.00				0.017
VRF-262	4.3	43,115				4.82				0.006

Table X			Fold Change (SKF(h) vs DMSO 24h)				P-Value, T-Test, (SKF(h) vs DMSO 24h)			
VRF	**PI**	**MW (Da)**	**SKF (1h)**	**SKF (2h)**	**SKF (4h)**	**SKF (24h)**	**SKF (1h)**	**SKF (2h)**	**SKF (4h)**	**SKF (24h)**
VRF-263	4.8	66,951				5.15				0.015
VRF-264	4.6	97,834				6.37				0.008
VRF-265	7.9	52,366				7.76				0.027
VRF-266	8.1	105,028				12.35				0.010
VRF-267	4.4	39,734				14.17				0.007
VRF-268	4.3	42,662				19.01				0.013
VRF-269	5.3	90,717				27.04				0.016
VRF-270	4.1	56,649				32.87				0.009
VRF-271	8.6	81,530				49.03				0.023
VRF-272	4.5	53,381				72.74				0.032

For some preferred VRFs, VRF-11, VRF-12, VRF-17, VRF-30, VRF-32, VRF-54, VRF-68, VRF-69, VRF-73, VRF-79, VRF-83, VRF-92, VRF-96, VRF-97, VRF-101, VRF-127, VRF-136, VRF-139, VRF-147, VRF-152, VRF-153, VRF-156, VRF-159, VRF-162, VRF-168, VRF-170, VRF-171, VRF-175, VRF-176, VRF-178, VRF-179, VRF-181, VRF-183, VRF-192, VRF-194, VRF-211, VRF-218, VRF-220, VRF-221, VRF-227, VRF-232, VRF-238, VRF-241, VRF-242, VRF-247, VRF-251, VRF-259, VRF-262, VRF-264, VRF-266, VRF-267, VRF-270, the difference was highly significant ($p < 0.01$), and for certain highly preferred VRFs, VRF-34, VRF-56, VRF-64, VRF-70, VRF-94, VRF-116, VRF-148, VRF-155, VRF-191, VRF-196, VRF-199, VRF-216, VRF-219, VRF-225, VRF-228, the difference was still more significant ($p < 0.001$).

The present invention is not to be limited in terms of the particular embodiments described in this application, which are intended as single illustrations of individual aspects of the invention. Functionally equivalent methods and apparatus within the scope of the invention, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications and variations are intended to fall within the scope of the appended claims. The contents of each reference, patent and patent application cited in this application is hereby incorporated by reference in its entirety.

When a reference is made herein to a method of treating or preventing a disease or condition using a particular agent or combination of agents, it is to be understood that such a reference is intended to include the use of that agent or combination of agents in the preparation of a medicament for the treatment or prevention of the disease or condition.

Preferred features of each aspect of the invention are as for each of the other aspects

mutatis mutandis.